DOI: 10.1534/genetics.109.103846

Collaboration Between the Essential Esa1 Acetyltransferase and the Rpd3 Deacetylase Is Mediated by H4K12 Histone Acetylation in *Saccharomyces cerevisiae*

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Manuscript received April 13, 2009
Accepted for publication July 7, 2009

ABSTRACT

Histone modifications that regulate chromatin-dependent processes are catalyzed by multisubunit complexes. These can function in both targeting activities to specific genes and in regulating genomewide levels of modifications. In *Saccharomyces cerevisiae*, Esa1 and Rpd3 have opposing enzymatic activities and are catalytic subunits of multiple chromatin modifying complexes with key roles in processes such as transcriptional regulation and DNA repair. Esa1 is an essential histone acetyltransferase that belongs to the highly conserved MYST family. This study presents evidence that the yeast histone deacetylase gene, *RPD3*, when deleted, suppressed *esa1* conditional mutant phenotypes. Deletion of *RPD3* reversed rDNA and telomeric silencing defects and restored global H4 acetylation levels, in addition to rescuing the growth defect of a temperature-sensitive *esa1* mutant. This functional genetic interaction between *ESA1* and *RPD3* was mediated through the Rpd3L complex. The suppression of *esa1*'s growth defect by disruption of Rpd3L was dependent on lysine 12 of histone H4. We propose a model whereby Esa1 and Rpd3L act coordinately to control the acetylation of H4 lysine 12 to regulate transcription, thereby emphasizing the importance of dynamic acetylation and deacetylation of this particular histone residue in maintaining cell viability.

THE genome of eukaryotic cells is packaged in chromatin, where the DNA is organized into a nucleosomal subunit structure. Nucleosomes consist of DNA wrapped around a histone octamer that contains two copies of each of the four core histones (H2A, H2B, H3, and H4), each of which can be post-translationally modified with multiple types of chemical and protein additions. The addition and removal of these modifications are catalyzed by histone modifying enzymes that function in a wide range of nuclear processes.

One dynamic histone modification is the acetylation and deacetylation of lysine residues. Enzymes that add an acetyl group to a lysine residue are known as histone acetyltransferases (HATs), and the enzymes that remove acetyl groups are called histone deacetylases (HDACs). The opposing activities of these two types of enzymes control the status of histone acetylation in the cell. For example, in the budding yeast *Saccharomyces cerevisiae*, regulation of H4 lysine 16 (H4K16) acetylation is critical in maintaining transcriptionally silent chromatin at the telomeres and regulating replicative life span, via activities of the HAT Sas2 and the HDAC Sir2 (Kimura *et al.* 2002; Suka *et al.* 2002; Dang *et al.* 2009).

Roles for the other acetylated lysines on H4 are less clearly defined. Some information has come from studying these modifications on a genomewide level. Through one microarray expression study, it became apparent that H4K5, H4K8, and H4K12 might contribute nonspecifically but jointly to transcription. Each lysine, when individually mutated to an unacetylatable amino acid, results in minimal changes in genomewide transcription. However, when combined to make double or triple lysine mutants, they display additive effects on transcription (Dion *et al.* 2005). In addition to participating in transcriptional control, H4 acetylation is critical for other nuclear processes, including DNA replication and repair (Megee *et al.* 1995; Bird *et al.* 2002; Choy and Kron 2002). Esa1 and Rpd3 are yeast enzymes with opposing activities toward H4 lysine acetylation, and are also members of two highly conserved families of histone modifying enzymes (reviewed in Doyon and Côté 2004; Yang and Seto 2008).

Rpd3 is one of the founding members of class I HDACs, which include the human proteins HDAC1, 2, 3, and 8 that are often overexpressed in human cancer cells. Indeed, HDAC inhibitors are being actively used and studied as therapeutic agents for multiple types of cancer (reviewed in Yang and Seto 2008). In yeast, Rpd3

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.103846/DC1.

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deacetylates lysines on both H3 and H4 (Rundlett *et al.* 1996) and is involved in a wide range of nuclear processes. On a global scale, Rpd3 is responsible for transcriptional regulation of a large number of genes (Bernstein *et al.* 2000; Sabet *et al.* 2004; Alejandro-Osorio *et al.* 2009). For many, but not all of these genes, transcriptional regulation occurs through modification of the lysines on the H3 and H4 N termini (Sabet *et al.* 2004). When examined at specific loci, Rpd3 represses transcription of *INO1* and *IME2* by deacetylating histones at the promoters of these genes (Kadosh and Struhl 1998b; Rundlett *et al.* 1998). In contrast, there are several transcripts that require Rpd3 for their activation (de Nadal *et al.* 2004; Sertil *et al.* 2007). For example, Rpd3 is required for expression of the DNA damage inducible genes *HUG1* and *RNR3* (Sharma *et al.* 2007). In line with Rpd3 having a role in DNA repair, *rpd3* mutants are defective in nonhomologous end joining (Jazayeri *et al.* 2004). Mutants of *rpd3* increase silencing at the telomeres, ribosomal DNA (rDNA) repeats, and *HM* cryptic mating-type loci (de Rubertis *et al.* 1996; Rundlett *et al.* 1996; Vannier *et al.* 1996; Sun and Hampsey 1999), although the mechanism for this is unknown. Rpd3 also contributes to cell cycle control, in that *rpd3* mutants undergo early DNA replication origin firing (Vogelauer *et al.* 2002; Aparicio *et al.* 2004).

Rpd3 exists in two biochemically defined complexes, named Rpd3S (small) and Rpd3L (large) to reflect their relative sizes (Carrozza *et al.* 2005b; Keogh *et al.* 2005). The identification of the subunits in each of the two Rpd3-containing complexes has begun to reveal a separation in Rpd3 complex functions. Rpd3L is likely responsible for Rpd3's role at gene promoters, as Rpd3 is recruited to chromatin via the Rpd3L-specific subunit Ume6, which recognizes specific upstream promoter sequences (Kadosh and Struhl 1997; Carrozza *et al.* 2005a). Rpd3L-specific mutants also display increased rDNA, telomeric, and *HM* loci silencing (Vannier *et al.* 1996; Zhang *et al.* 1998; Sun and Hampsey 1999; Loewith *et al.* 2001; Carrozza *et al.* 2005a; Keogh *et al.* 2005), and replication timing defects (Knott *et al.* 2009) similar to *rpd3*Δ itself, indicating that Rpd3L is responsible for Rpd3's role in silencing and regulation of replication initiation. The smaller Rpd3S complex is recruited to methylated H3K36 within coding sequences to repress intragenic transcription initiation (Carrozza *et al.* 2005b; Keogh *et al.* 2005), a role not shared by Rpd3L.

Whereas Rpd3 is a class I HDAC, Esa1 is part of the evolutionarily conserved MYST family of HATs. Tip60, the human homolog of Esa1, is associated with many human diseases, including HIV, Alzheimer's and multiple cancers. Tip60 acetylates the tumor suppressor p53 and acts as a transcriptional coactivator for c-Myc and NF-κB (reviewed in Avvakumov and Côté 2007). In yeast, Esa1 is essential for viability (Smith *et al.* 1998; Clarke *et al.* 1999), although Esa1's essential function may not be limited to its catalytic activity (Decker *et al.* 2008). Esa1 acetylates specific lysine residues on histones H2A, H3, H4 (Smith *et al.* 1998; Clarke *et al.* 1999), and the histone variant H2A.Z (Babiarz *et al.* 2006; Keogh *et al.* 2006; Millar *et al.* 2006). Similar to Rpd3, Esa1 is the catalytic subunit of two chromatin modifying complexes that have shared subunits. The larger of these is known as NuA4, whereas the smaller is piccolo (Allard *et al.* 1999; Boudreault *et al.* 2003). *In vitro* activity assays indicate that piccolo is the more active acetyltransferase complex on chromatin, but separate roles for the two complexes have not been established *in vivo* (Boudreault *et al.* 2003).

Through the study of conditional *esa1* mutants, Esa1 has been discovered to play an important role in many nuclear processes. Esa1 functions in cell cycle progression (Clarke *et al.* 1999) and DNA repair (Bird *et al.* 2002). Esa1 also contributes both to transcriptional activation and repression. Esa1 binding has been observed at the promoters of many transcriptionally active genes (Robert *et al.* 2004), and specifically at ribosomal protein genes, where Esa1 is needed for their transcriptional activation (Reid *et al.* 2000). In a somewhat opposing role, Esa1 is required for transcriptional silencing at the telomeres and the rDNA. Specifically, Pol II reporter genes that are normally repressed when inserted at the telomeres and the rDNA display increased expression in *esa1* mutants (Clarke *et al.* 2006).

In this study, we defined an *in vivo* collaboration between the histone acetyltransferase Esa1 and the histone deacetylase Rpd3. Genetic dissection of the functional interactions revealed that the collaboration is mediated specifically through the Rpd3L complex. Deletion of *RPD3* suppressed multiple phenotypes of *esa1* mutants, including temperature sensitivity, rDNA and telomeric silencing defects, and restored global H4 acetylation defects. Deletion of genes encoding Rpd3L-specific subunits Pho23 or Sds3 likewise promoted suppression of *esa1* phenotypes, suggesting that Esa1 coordinates acetylation specifically with Rpd3L. Consistent with this interpretation, deletion of the Rpd3S subunit encoded by *RCO1* did not suppress *esa1* phenotypes. Finally, suppression of the growth defect in the *esa1* mutant by deletion of Rpd3L subunits was specifically dependent on acetylation of lysine 12 on histone H4, thereby pointing to a crucial yet previously unsuspected role for this specific residue.

MATERIALS AND METHODS

Yeast strains and plasmids: All strains and plasmids are listed in supporting information, Table S1 and Table S2. Except where noted with specific allele designations, all mutations used are null alleles constructed using standard molecular methods. The *rpd3::kanMX* (LPY12154), *hda1::kanMX* (LPY13472), *hos1::kanMX* (LPY13706), and *hos2::kanMX* (LPY13583) mutants were constructed using marker

swap plasmids M3926, M3929, and M3925 as described in Voth *et al.* (2003) on *rpd3::LEU2* (DY1539) (Kasten *et al.* 1997), *hda1::TRP1* (DY4891), *hos1::HIS3* (DY6073), *hos2::TRP1* (DY4549) (all generous gifts from D. Stillman) and then backcrossed prior to use. The *pho23Δ::kanMX* (LPY12732), *sds3Δ::kanMX* (LPY12958), and *rco1Δ::kanMX* (LPY12645) mutants were constructed by amplification (oligonucleotides listed in Table S3) of the *kanMX* cassettes from Saccharomyces Genome Deletion Project strains, transformed into W303-1a (LPY5) and backcrossed prior to use. All double mutants and the silencing markers rDNA*::ADE2-CAN1* (Fritze *et al.* 1997) and TELVR*::URA3* (Renauld *et al.* 1993) were introduced through standard genetic crosses. Construction of the *RPD3* catalytic mutant plasmid, *rpd3-H150A-H151A*, is described in Ruault and Pillus (2006). Histone mutant strains, derived from MSY1905 (a generous gift from M. M. Smith) (Ruault and Pillus 2006) are chromosomally deleted for both *HHF-HHT* loci and initially contained wild-type histones on the plasmid pJH33 (*CEN URA3 HTA1 HTB1 HHF2 HHT2*) (Ahn *et al.* 2005). For mutant construction, strains were transformed with a *TRP1* plasmid containing the relevant H4 (*HHF2*) mutation, and then subjected to a plasmid shuffle by counterselection on 5-FOA to remove pJH33. Histone mutant plasmids were constructed by site-specific mutagenesis using oligonucleotides listed in Table S3.

Growth dilution assays and silencing assays: Unless otherwise noted, all dilution assays represent fivefold serial dilutions, starting from an A_{600} of 1.0 after growth to saturation in 3 ml of liquid synthetic complete (SC) medium. Growth and silencing plates were incubated at 30°. Suppression of *esa1*'s growth defect on SC plates was assayed at the restrictive temperature of 35°. The rDNA silencing assays were performed with strains containing the rDNA*::ADE2-CAN1* reporter as described (van Leeuwen and Gottschling 2002). Strains were grown in SC−Ade−Arg medium to saturation, normalized as above, and plated on SC−Ade−Arg (growth control) and SC−Ade−Arg plates containing 32 μg/ml of canavanine (to assay rDNA silencing). Telomeric silencing assays were performed with the TELVR*::URA3* reporter as described (Renauld *et al.* 1993; van Leeuwen and Gottschling 2002). Strains were grown in SC medium and plated on both SC (growth control) and SC with 0.1% 5-FOA (to assay telomeric silencing). Camptothecin (CPT) sensitivity was assayed using CPT dissolved in DMSO added to plates buffered with 100 mm potassium phosphate (pH 7.5) to maintain maximal drug activity (Nitiss and Wang 1988). Growth control plates contained equal concentrations of DMSO and phosphate buffer. All images were captured after 2–4 days of growth.

Protein immunoblots: Whole cell extracts were prepared from cells grown to A_{600} of 0.6–1.0 at 30°. In the temperature-shift experiment, cells were grown first at 30° and then shifted to 34° for six hours. Extracts were prepared by bead beating as described previously (Clarke *et al.* 1999). Briefly, cells were resuspended in phosphate buffered saline with protease inhibitors and lysed by vortexing with glass beads. Whole cell extracts were then denatured by boiling in sample loading buffer and separated from the insoluble pellet by centrifugation. Proteins were separated on 18% SDS-polyacrylamide gels and transferred to nitrocellulose (0.2 μm). Primary antisera used were anti-H4K5Ac (1:5000 dilution, Serotec), anti-H4K8Ac (1:2500 dilution, Serotec), anti-H4K12Ac (1:2500 dilution, Serotec), anti-H4K16Ac (1:2500 dilution, Upstate), and anti-H3 CT (1:10,000 dilution, Upstate). Goat anti-rabbit conjugated to horseradish peroxidase (Promega) was used as a secondary antibody, and signal was detected with Western Lightning Chemiluminescence Reagent (Perkin Elmer) on Kodak X-Omat film.

RESULTS

Deletion of the histone deacetylase gene *RPD3* suppressed the growth defect of *esa1*: Histone acetylation and deacetylation are opposing chemical modifications that must be balanced for transcriptional regulation. The interplay between HATs and HDACs is complicated by the presence of numerous enzymes, some of which have very specific substrates, whereas others share overlapping histone targets. In attempting to dissect these relationships, large-scale studies have uncovered an intricate network of genetic interactions between multiple HATs and HDACs (Collins *et al.* 2007; Lin *et al.* 2008; Mitchell *et al.* 2008).

In a study directly examining Esa1's functions in rDNA silencing, a surprising relationship was discovered between Esa1 and the class III HDAC Sir2 (Clarke *et al.* 2006). When either *ESA1* or *SIR2* is overexpressed, each suppresses the rDNA silencing defects of the other mutant. For example, overexpression of *ESA1* rescues the rDNA silencing defect of the *sir2* mutant (Clarke *et al.* 2006).

Neither increased dosage nor deletion of *SIR2*, however, had any effect on the growth defect of the *esa1-414* temperature-sensitive mutant at elevated temperatures. In searching for other genes encoding chromatin modifying enzymes that might functionally interact with *esa1* mutants, deletion of *RPD3* was discovered to specifically suppress this growth defect (Figure 1).

To ask whether deletion of genes encoding other HDACs could also support viability of the temperature-sensitive *esa1-414* allele at restrictive temperatures, double mutants of *esa1-414* were constructed in combination with a series of HDAC mutants. Along with the class III family deacetylase Sir2, deletion of genes encoding HDACs of classes I and II were tested. Rpd3, Hos1, Hos2, and Hda1 are all yeast HDACs of classes I and II that share 25–50% protein sequence identity. Of these, only *RPD3* deletion supported growth of *esa1* mutants at elevated temperatures (Figure 1). Mutation of the other genes either had no effect or in the case of *hos2*, exacerbated the severity of the *esa1* growth defect. Some of these results are parallel to interactions reported in a genomewide study (Lin *et al.* 2008).

The *esa1-414* temperature-sensitive mutant contains a frameshift mutation that results in an early truncation of the protein and displays reduced HAT activity both *in vitro* and *in vivo* (Clarke *et al.* 1999). To test the allele specificity of the suppression, *RPD3* was deleted in another *esa1* temperature-sensitive allele, *esa1-L254P*, and was also found to suppress the *esa1* growth defect at restrictive temperature (Figure S1, A). The *esa1-L254P* allele contains a point mutation that resides near the HAT domain, and similar to *esa1-414*, is temperature sensitive and lacks *in vitro* and *in vivo* HAT activity (Clarke *et al.* 1999). Thus, *rpd3* suppression of the *esa1* growth defect is not allele specific and may be a general

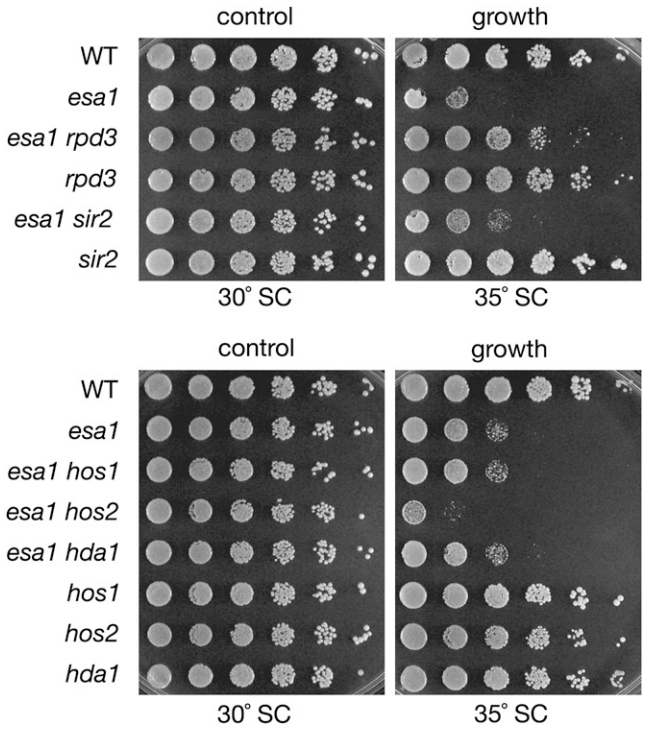

Figure 1.—Deletion of the histone deacetylase gene *RPD3* suppresses the growth defect of *esa1*. Deletion of *RPD3* suppressed the temperature sensitivity of the *esa1-414* allele, whereas deletion of other genes encoding histone deacetylases did not. Top panel: serial dilutions of wild type (LPY5), *esa1* (LPY4774), *esa1 rpd3* (LPY12156), *rpd3* (LPY12154), *esa1 sir2* (LPY11160), and *sir2* (LPY11) are shown on SC at the restrictive temperature for *esa1* (35°), compared to growth at the permissive temperature (30°). Bottom panel: serial dilutions of wild type (LPY5), *esa1* (LPY4774), *esa1 hos1* (LPY13712), *esa1 hos2* (LPY13585), *esa1 hda1* (LPY13478), *hos1* (LPY13706), *hos2* (LPY13583), and *hda1* (LPY13472) on SC at restrictive and permissive temperatures. Note that some of these interactions overlap with published results from a genomewide study (Lin *et al.* 2008), yet others are distinct. The differences may be due to strain background- or allele-specific effects (Y. Lin and J. Boeke, personal communication).

property for catalytically compromised Esa1. Furthermore, using the *RPD3* catalytically dead allele, *rpd3-H150A-H151A* (Kadosh and Struhl 1998a) in combination with the *esa1* mutant showed results consistent with the *rpd3*Δ (Figure S1, B). Therefore, the growth rescue observed is due to loss of histone deacetylase activity by Rpd3, and not some other function of Rpd3.

To test whether *RPD3* deletion could bypass the nonviable *esa1*Δ phenotype, two tests were conducted. In the first, a plasmid shuffle was performed with a wild-type *ESA1* plasmid in the *esa1*Δ *rpd3*Δ double mutant. This strain was unable to grow under conditions that select for loss of the wild-type *ESA1* plasmid (Figure S1, C). In the second test, an *esa1*Δ*/ESA1 rpd3*Δ*/rpd3*Δ diploid was sporulated, dissected, and examined for viability. All genotypically *esa1*Δ *rpd3*Δ double mutants were inviable. Some double mutants germinated and were able to undergo a small number of divisions, but none continued dividing to form colonies (data not shown), similar to *esa1*Δ itself (Clarke *et al.* 1999). This analysis confirmed the plasmid shuffle result, demonstrating that *rpd3* did not suppress the inviable *esa1*Δ.

Suppression of *esa1*'s growth defect was mediated exclusively by the Rpd3L complex: Rpd3S and Rpd3L, the two Rpd3-containing HDAC complexes, each have shared subunits as well as a number of distinct subunits (Carrozza *et al.* 2005a,b; Keogh *et al.* 2005) (Figure 2A). Both Rpd3S and Rpd3L also contain proteins that function in additional nuclear complexes. By evaluating subunits that are unique to Rpd3S and Rpd3L, the complexes were dissected genetically to determine whether deletion of both is required, or whether instead the suppression observed in the *esa1 rpd3* double mutant is mediated through one specific complex. Double mutants were constructed with *esa1* and genes specific to each of the two complexes. These double mutants were then tested for suppression of *esa1*.

Rpd3S, the smaller of the two complexes, has only two subunits (Eaf3, Rco1) that distinguish it from Rpd3L (Carrozza *et al.* 2005b; Keogh *et al.* 2005). However, Eaf3 is not unique to Rpd3S since it is also a component of NuA4 (Eisen *et al.* 2001), an Esa1-containing complex. Loss of *EAF3* disrupts both NuA4 and Rpd3S, thus *RCO1* was chosen instead to disrupt Rpd3S. The Rco1 protein contains a PHD finger and is required for the complex integrity of Rpd3S (Carrozza *et al.* 2005b). As shown in Figure 2B (top panel), deletion of *RCO1* in an *esa1* mutant did not suppress the *esa1* growth defect. In fact, the *esa1 rco1* double mutant displayed a slightly exacerbated growth defect compared to that of *esa1*. Therefore, the suppression of *esa1*'s growth defect is not mediated through Rpd3S.

Rpd3L contains several subunits distinct from those in Rpd3S. Some are involved in the function of other transcriptional complexes, such as Cti6, which recruits the SAGA HAT complex to chromatin for transcriptional activation (Papamichos-Chronakis *et al.* 2002). In contrast, Sds3 is a subunit unique to Rpd3L. Sds3 is essential for the integrity of the Rpd3L complex, and Rpd3L dissociates in *sds3* mutants, thereby resulting in a loss of all Rpd3L histone deacetylase activity (Lechner *et al.* 2000; Carrozza *et al.* 2005a). Deletion of *SDS3* in an *esa1* mutant mimicked the suppression seen in *esa1 rpd3* (Figure 2B, bottom), providing evidence that suppression of *esa1* is mediated through Rpd3L.

Pho23 is another Rpd3L-specific protein with a PHD finger and is one of three yeast proteins that belong to the ING tumor suppressor family (Loewith *et al.* 2001). In contrast to the *sds3*Δ mutant, the Rpd3L complex is structurally intact in *pho23*Δ cells and has normal levels of *in vitro* histone deacetylase activity (Carrozza *et al.* 2005a). Deletion of *PHO23* in the *esa1* mutant mimicked the suppression seen in both the *esa1 rpd3* and *esa1 sds3*

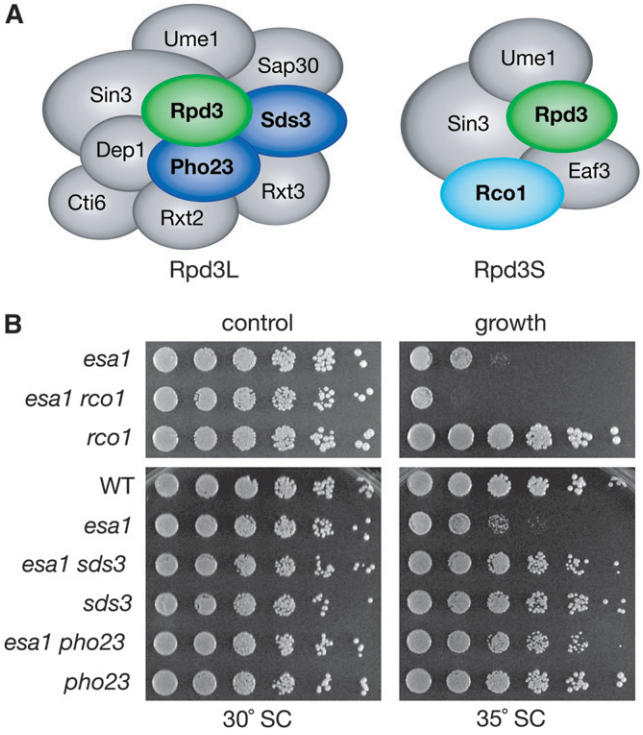

Figure 2.—Rpd3L is the Rpd3-containing complex responsible for suppression of the growth defect in the *esa1* mutant. (A) Cartoon highlighting the unique and shared members of the Rpd3S and Rpd3L complexes. (B) Deletion of *RCO1*, specific to Rpd3S, did not suppress *esa1*'s growth defect at restrictive temperature. Deletion of *PHO23* and *SDS3*, both specific to Rpd3L, mimicked the suppression seen in *esa1 rpd3*. Serial dilutions of wild type (LPY5), *esa1* (LPY4774), *esa1 rco1* (LPY12652), *rco1* (LPY12645), *esa1 sds3* (LPY12956), *sds3* (LPY12958), *esa1 pho23* (LPY12729), and *pho23* (LPY12732), were plated on SC at permissive (30°) and restrictive temperatures (35°). Cartoon of complexes is modified from Roguev and Krogan (2007).

double mutants (Figure 2B, bottom). This minor disruption in the Rpd3L complex is able to suppress *esa1*'s growth defect and supports the idea that Pho23 may have a key targeting function that works in opposition to other PHD finger proteins that exist in NuA4 and piccolo.

Comparing the growth at elevated temperatures of *esa1 rco1* mutants to the *esa1 sds3* and *esa1 pho23* strains thus demonstrates that the rescue of *esa1*'s growth defect by deletion of *RPD3* is mediated by the Rpd3L complex and not by Rpd3S. This specificity of suppression further establishes functional and not merely structural distinctions between the two Rpd3 complexes. To determine whether the specificity of suppression extended to the diverse biological roles of Esa1, a broader analysis of defective *esa1* functions was evaluated.

Disruption of Rpd3L suppressed silencing phenotypes of the *esa1* mutant: Mutants of *ESA1* have a wide range of phenotypes, including defects in cell cycle control, transcriptional silencing, and the DNA damage response (Clarke *et al.* 1999; Bird *et al.* 2002; Clarke *et al.* 2006). To determine the involvement of Rpd3L in contributing to these phenotypes, the *esa1 rpd3* double mutants along with the complex-specific double mutants were examined for the integrity of these functions using *in vivo* assays.

First, rDNA silencing was assayed in the *esa1 rpd3* double mutant. Rpd3 has a previously reported increase in rDNA silencing (Sun and Hampsey 1999), confirmed here with the observation that *rpd3* increased repression of a *CAN1* reporter integrated at a single 25S rDNA locus (Figure 3A). In contrast, *esa1* mutants are defective in silencing at the rDNA (Clarke *et al.* 2006) (Figure 3A). Deletion of *RPD3* in combination with *esa1* suppressed this rDNA silencing defect. Deletion of *RPD3* in the *esa1* mutant not only rescued the rDNA silencing defect, but increased silencing in the double mutant to that seen in an *rpd3* single mutant (Figure 3A, top). This same trend was observed when the catalytic residues of *RPD3* were mutated in combination with *esa1* (Figure S2, A). The complex-specific double mutants were next tested for rDNA silencing. The previous observations that Rpd3L-specific mutants display increased rDNA silencing (Sun and Hampsey 1999; Loewith *et al.* 2001; Keogh *et al.* 2005) were confirmed. Consistent with the suppression of *esa1*'s growth defect, rDNA silencing was suppressed only when Rpd3L was disrupted in *esa1* and not when Rpd3S was disrupted (Figure 3A, compare *sds3* and *pho23* to *rco1* mutants).

Telomeric silencing was next assayed and revealed suppression patterns parallel to those for rDNA silencing. The *esa1* mutant is defective in silencing a *URA3* reporter gene integrated at telomere VR (Clarke *et al.* 2006) (Figure 3B). The *rpd3* and Rpd3L-specific mutants displayed increased silencing (Figure 3B), confirming previous reports (Vannier *et al.* 1996; Zhang *et al.* 1998; Loewith *et al.* 2001; Carrozza *et al.* 2005a; Keogh *et al.* 2005). When genes encoding Rpd3L subunits were deleted in combination with *esa1*, they all restored telomeric silencing to *esa1* mutants, whereas deletion of the Rpd3S-specific *RCO1* had no effect on *esa1*'s reduced telomeric silencing (Figure 3B and Figure S2, B). Therefore, Esa1 and Rpd3L share a critical opposing role in silencing at both the rDNA and telomeres.

Rpd3L disruption did not suppress the DNA damage phenotype of the *esa1* mutant: Whereas the growth and silencing phenotypes of *esa1* are consistent with Esa1's transcriptional functions, Esa1 also has a role in DNA double-strand break repair. This is readily observed in that *esa1* mutants are sensitive to camptothecin (Bird *et al.* 2002), a phenotype associated with defects in DNA repair and genome integrity. Camptothecin causes double-strand breaks by inhibiting topoisomerase I (Hsiang *et al.* 1985). Rpd3 also contributes to double-strand break repair, and *rpd3* mutants are sensitive to phleomycin, another DNA damaging agent (Jazayeri *et al.* 2004).

When tested in plate assays, an *rpd3* single mutant displayed increased sensitivity to camptothecin, and

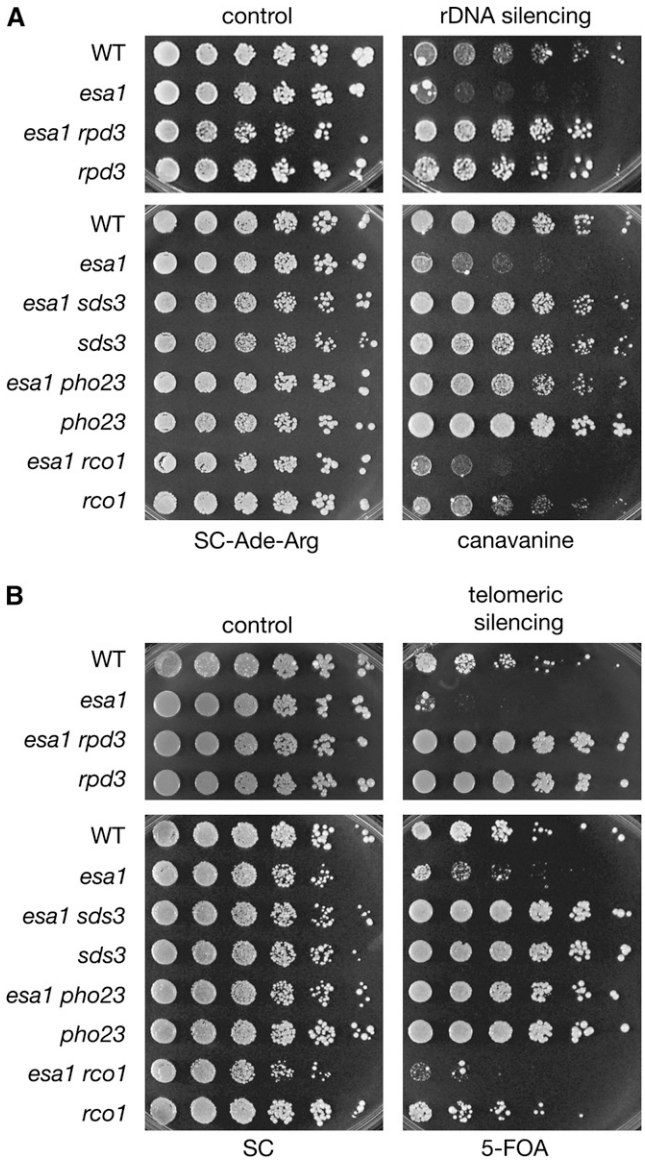

Figure 3.—Disruption of Rpd3L suppresses *esa1* silencing phenotypes. (A) Disruption of Rpd3L suppressed the rDNA silencing defect of an *esa1* mutant. Wild type (LPY4909), *esa1* (LPY4911), *esa1 rpd3* (LPY12147), *rpd3* (LPY12145), *esa1 sds3* (LPY13517), *sds3* (LPY13513), *esa1 pho23* (LPY13859), *pho23* (LPY13854), *esa1 rco1* (LPY13505), and *rco1* (LPY13501) all have the rDNA::*ADE2-CAN1* reporter to test for rDNA silencing on plates containing canavanine. Decreased growth on canavanine indicates a defect in rDNA silencing. (B) Disruption of Rpd3L suppressed the telomeric silencing defect of an *esa1* mutant. Wild type (LPY4917), *esa1* (LPY4919), *esa1 rpd3* (LPY12211), *rpd3* (LPY12093), *esa1 sds3* (LPY13540), *sds3* (LPY13536), *esa1 pho23* (LPY13769), *pho23* (LPY13765), *esa1 rco1* (LPY13528), and *rco1* (LPY13524) all have the TELVR::*URA3* silencing marker to test for telomeric silencing on plates containing 5-FOA. Decreased growth on 5-FOA indicates a defect in telomeric silencing.

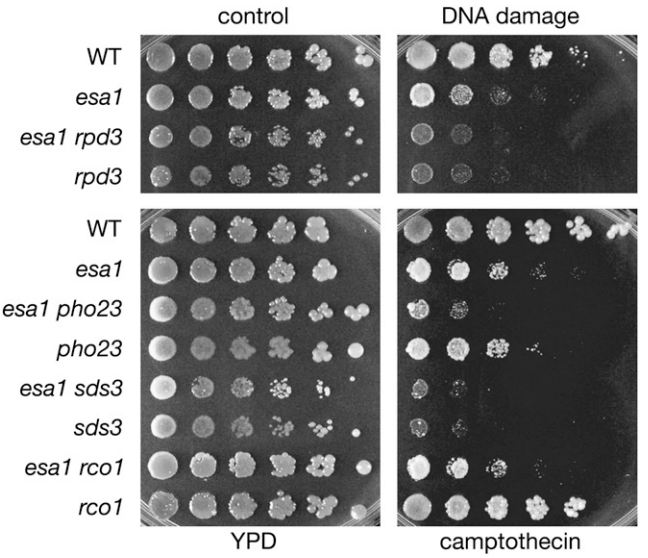

Figure 4.—Rpd3L disruption does not suppress *esa1*'s camptothecin sensitivity. The same strains tested in Figure 1 and Figure 2B were plated on a control YPD plate containing DMSO and a plate containing 20 μg/ml of camptothecin in DMSO.

rpd3 did not suppress *esa1*'s sensitivity to camptothecin (Figure 4). In fact, the *esa1 rpd3* double mutant had increased sensitivity to camptothecin compared to *esa1* alone. Deletion of Rpd3L- and Rpd3S-specific subunits either exacerbated or had a minimal effect on camptothecin sensitivity in the *esa1* mutant (Figure 4).

Esa1 and Rpd3 are among several chromatin modifiers that are recruited to the repair of double-strand breaks resulting from DNA damage (Downs *et al.* 2004; Tamburini and Tyler 2005; Lin *et al.* 2008). Camptothecin sensitivity in *esa1* cells is thought to result from a failure of Esa1 and NuA4 recruitment to double-strand breaks. Therefore, *rpd3* as a suppressor of *esa1* is unlikely to involve Esa1's role in acetylation at sites of DNA damage.

In addition to the silencing and DNA damage phenotypes, *rpd3* mutants have reduced mating efficiency and are cycloheximide sensitive (Vidal and Gaber 1991). To examine whether mutation of *ESA1* could reciprocally suppress *rpd3* phenotypes, the *esa1 rpd3* and the complex-specific double mutants were examined for changes in mating efficiency and cycloheximide sensivitity. Mutation of *ESA1* in *rpd3* had no effect on the reduced mating efficiency of *rpd3*, and the same was seen for the Rpd3L-specific mutants *pho23* and *sds3* (Figure S3, A). The mating defect of *rpd3* mutants appeared specific to Rpd3L, shown by the reduced mating of *pho23* and *sds3* compared to wild-type mating in *rco1* (Figure S3, A). Reduced mating efficiency has been observed previously for *sap30*, another Rpd3L-specific mutant (Zhang *et al.* 1998).

To determine whether mutation of *ESA1* could suppress *rpd3*'s cycloheximide sensitivity, growth of the *esa1 rpd3* double mutant was examined on cycloheximide-containing plates. No suppression was observed; in fact the *esa1* mutant displayed a previously unreported modest cycloheximide resistance (Figure S3, B). Together, *rpd3* in the context of Rpd3L can suppress

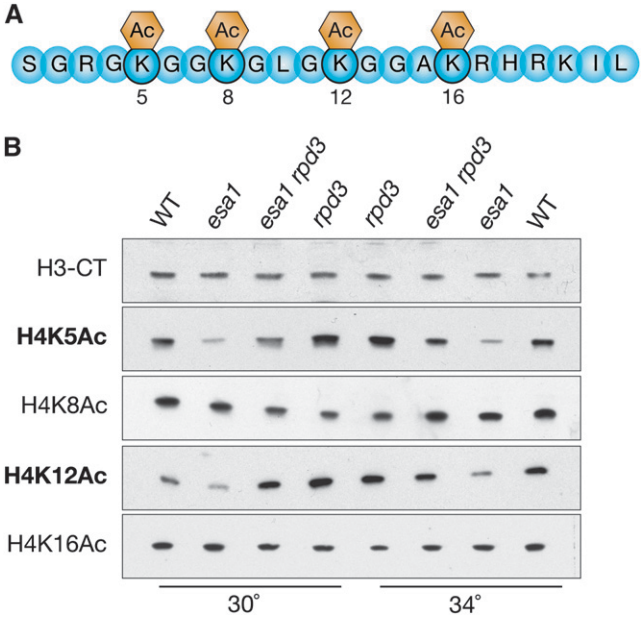

FIGURE 5.—Deletion of *RPD3* restores global acetylation levels of specific histone H4 residues in *esa1* mutants. (A) Diagram of the histone H4 N-terminal tail highlighting sites of acetylation modifications. (B) Deletion of *RPD3* restored global acetylation of H4K5 and H4K12, but not H4K8 and H4K16. Whole cell protein extracts from wild-type (LPY5), *esa1* (LPY4774), *esa1 rpd3* (LPY12156), and *rpd3* (LPY12154) cells at both permissive (30°) and restrictive (34°) temperatures were immunoblotted with an antiserum specific to the C terminus of H3 to control for histone levels, and with H4 antisera to detect the amount of bulk histone acetylation at each lysine residue.

many but not all *esa1* mutant phenotypes. The nature of this functional interaction is not however reciprocal because *esa1* mutants do not suppress the *rpd3* defects tested.

Deletion of *RPD3* restored global histone acetylation levels of shared target residues in the *esa1* mutant: To test whether the genetic relationship discovered between *esa1* and *rpd3* was observed at the molecular level of histone modification, global histone acetylation was evaluated using isoform-specific antisera for lysines targeted by Esa1 and Rpd3. The enzymatic activities of Esa1 and Rpd3 both target specific lysines on the N-terminal tail of histone H4 (Figure 5A). Esa1 has global effects *in vivo* on H4K5 acetylation (CLARKE *et al.* 1999) and also acetylates multiple lysines on H4 at sites within the rDNA (CLARKE *et al.* 2006) and at specific gene promoters (SUKA *et al.* 2001). Rpd3 globally deacetylates H4K5 and H4K12 (RUNDLETT *et al.* 1996) and is responsible for deacetylation of most histone tail lysine residues at specific gene promoters (SUKA *et al.* 2001).

Lysates were collected from cells grown at permissive and slightly elevated temperatures that maintained viability. Immunoblots were performed on these lysates and probed with antisera specific for each acetylated lysine. As a control, histone levels were assayed and found comparable among all strains, as shown by probing for the C terminus of H3 (Figure 5B, top panel). As expected, *esa1* mutants displayed decreased bulk histone acetylation, most dramatic for H4K5 (CLARKE *et al.* 1999) and H4K12, and *rpd3* mutants had slightly increased acetylation of H4K5 and H4K12 compared to wild type, consistent with earlier reports (RUNDLETT *et al.* 1996) (Figure 5B). In the noncatalytic mutants (*rco1*, *sds3*, and *pho23*), global histone acetylation changes were not observed (Figure S4). This result might be expected since an Rpd3 complex is still present in each of these mutants. Thus, it was not surprising that there were only very subtle changes in acetylation in these mutants when combined with *esa1* (Figure S4). Additionally, at two H4 lysines that are not shared targets of Esa1 and Rpd3, H4K8 and H4K16, acetylation was not changed in the *esa1 rpd3* double mutant.

Finally, in the *esa1 rpd3* double mutant, there was almost a complete restoration of the *esa1* global acetylation defect at both permissive and elevated temperatures that was strongest for H4K12 (Figure 5B). There was also an intermediate effect on acetylation at H4K5. Together, these results provide a molecular basis for the growth defect and silencing suppression observed in the *esa1 rpd3* double mutant (Figures 1 and 3).

Suppression of *esa1*'s growth defect by deletion of *RPD3* is mediated through H4K12 acetylation: Because the most dramatic change in global histone acetylation in *esa1 rpd3* was at H4K12 (Figure 5B), it seemed likely that this particular residue was most critical for the functional interaction between the two enzymes. To evaluate the possibility, mutants were constructed in which each target lysine was changed to alanine, an amino acid residue that cannot be acetylated. The ability of *rpd3* to suppress *esa1*'s growth defect was then tested with each histone lysine mutant. In wild-type cells, the H4K12A mutant by itself did not display any growth defects, nor did it affect growth in the *esa1* or *rpd3* single mutant. However, H4K12A in combination with the *esa1 rpd3* double mutant displayed a dramatic reduction in growth at elevated temperature compared to the *esa1 rpd3* double mutant (Figure 6A, top). The other H4 lysine mutants (H4K5A, H4K8A, and H4K16A) had minimal effects on the growth of the *esa1 rpd3* double mutant (Figure 6A, bottom). The dependence on H4K12 was also observed in the *esa1 rpd3-H150A-H151A* catalytic mutant (Figure S5). Therefore, the suppression observed in the *esa1 rpd3* double mutant is specifically dependent on H4K12 and not H4K5, K8, or K16.

H4K12 was also found to be the key acetylated lysine in suppression of *esa1* by disruption of the Rpd3L complex. As shown in Figure 6B, when each lysine was individually mutated in the *esa1 sds3* and *esa1 pho23* double mutants, only the H4K12A substitution resulted in a loss of suppression, albeit to a more modest degree than in *esa1 rpd3*. Although the dependence on H4K12 appears subtle in the *esa1 pho23* double mutant, this slight effect was observed reproducibly. Notably, in the

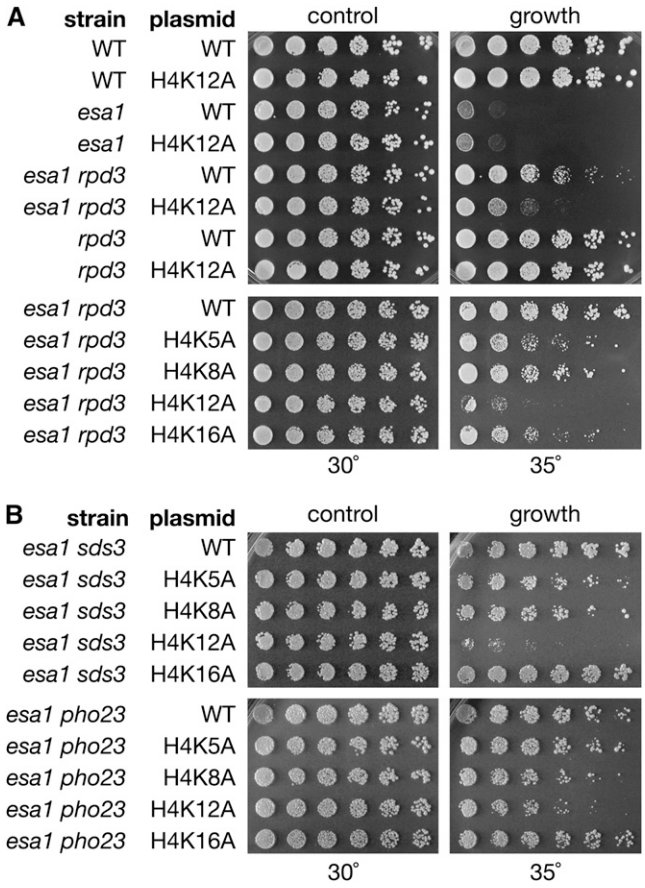

FIGURE 6.—Suppression of *esa1*'s growth defect by *rpd3* is dependent on H4K12. Strains are deleted for all copies of H3 and H4 and carry a *TRP1* plasmid with either wild-type H4 or H4 with one mutated lysine residue. Plasmid retention was required for survival. (A) Serial dilutions of the following strains were plated at permissive (30°) and restrictive temperatures (35°) on SC. Top panel: growth of H4K12A mutants in combination with *esa1 rpd3*. Strains are wild type (LPY12383), H4K12A (LPY12394), *esa1* (LPY12384), *esa1* H4K12A (LPY12071), *esa1 rpd3* (LPY12707), *esa1 rpd3* H4K12A (LPY12714), *rpd3* (LPY12695), and *rpd3* H4K12A (LPY12702). Bottom panel: growth of *esa1 rpd3* mutants in combination with each lysine individually mutated to alanine. Strains are *esa1 rpd3* (LPY12707), *esa1 rpd3* H4K5A (LPY12708), *esa1 rpd3* H4K8A (LPY12711), *esa1 rpd3* H4K12A (LPY12714), and *esa1 rpd3* H4K16A (LPY12717). (B) Suppression of *esa1*'s growth defect through deletion of noncatalytic Rpd3L subunits was also dependent on H4K12. Top panel: twofold dilutions, starting at an A_{600} of 0.1, were plated on SC−Trp for assaying growth of *esa1 sds3* in combination with each lysine individually mutated to alanine. Strains are *esa1 sds3* (LPY14175), *esa1 sds3* H4K5A (LPY14176), *esa1 sds3* H4K8A (LPY14177), *esa1 sds3* H4K12A (LPY14178), and *esa1 sds3* H4K16A (LPY14179). Bottom panel: as above except in *esa1 pho23* mutant. Strains are *esa1 pho23* (LPY14165), *esa1 pho23* H4K5A (LPY14166), *esa1 pho23* H4K8A (LPY14167), *esa1 pho23* H4K12A (LPY14168), and *esa1 pho23* H4K16A (LPY14169).

protein immunoblots H4K5 showed a moderate acetylation increase in the *esa1 rpd3* double mutant compared to *esa1* (Figure 5B), yet the H4K5A mutant had little impact on the growth of *esa1 rpd3*, *esa1 sds3*, or *esa1 pho23* (Figure 6).

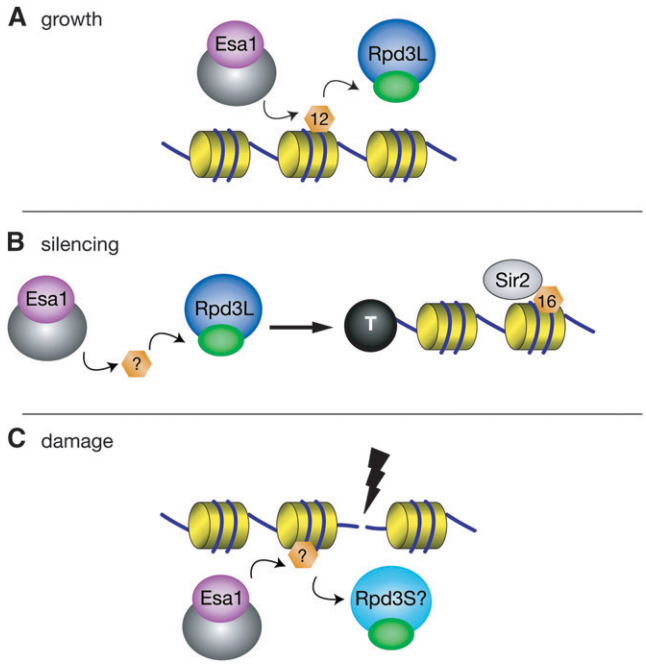

FIGURE 7.—A model depicting a critical role for Esa1 and Rpd3L in coordinating the dynamic acetylation of H4K12. (A) Esa1 and Rpd3L control H4K12Ac for general transcriptional targets contributing to cell viability and growth. (B) Esa1 and Rpd3L contribute to rDNA and telomeric silencing. This relationship is not mediated specifically through H4K12 acetylation, but likely through a number of other targets. Sir2 deacetylation of H4K16 appears downstream of the role for Esa1 and Rpd3L. (C) Esa1 and Rpd3S, but not Rpd3L, may control acetylation at sites of DNA damage.

Thus H4K5 and H4K12 are common targets of global acetylation and deacetylation by both Esa1 and Rpd3. However, the distinction observed here between the growth of *esa1 rpd3* in H4K5A *vs.* H4K12A mutants points to H4K12 as the critical shared target of Esa1 and the Rpd3L complex for regulating growth and viability (Figure 7A).

DISCUSSION

The findings presented here tightly link Esa1's acetyltransferase activity and Rpd3's deacetylase activity in critical cellular processes. Loss of Rpd3L specifically alleviated many of the cell's needs for fully functional Esa1 activity, a property not shared by the Rpd3S complex, nor by other class I–III deacetylases (summarized in Table S4). This exclusive relationship between Esa1 and Rpd3L centers on their shared histone target H4K12. In addition, Esa1 works specifically with the Rpd3L complex in maintaining silencing at the rDNA and telomeres, but not in repairing camptothecin-induced double-strand breaks (Figure 7, Table S4).

Esa1 and Rpd3 have both previously been shown to be required for rDNA and telomeric silencing. Esa1 is enriched at the rDNA by chromatin immunopreceiptation

and Esa1-dependent changes in H4 acetylation are seen at the rDNA (Clarke *et al.* 2006). Unlike its role in growth, the *rpd3*-mediated suppression of *esa1*'s rDNA and telomeric silencing defects was not dependent on H4K12 acetylation (Figure S6). Hence suppression at these loci is mechanistically distinct. Rpd3L's role in rDNA and telomeric silencing involves boundary formation (Zhou *et al.* 2009) and is dependent on the histone deacetylase Sir2 that targets H4K16 (Sun and Hampsey 1999; Raisner and Madhani 2008). Therefore the observed dependence on H4K16 acetylation (Figure S6) was not surprising. This dependence on Sir2 and H4K16 deacetylation has led to the idea that Rpd3 has an indirect effect in silencing, possibly through altering Sir2 activity (Sun and Hampsey 1999) or the expression of other genes involved in silencing. Because *esa1*'s rDNA and telomeric silencing defects were suppressed by disruption of Rpd3L (Figure 3), and were dependent on H4K16 acetylation (Figure S6), it is likely that Esa1 and Rpd3L's role in silencing is upstream of Sir2 (Figure 7B).

It is becoming evident that histone modifying enzymes also target many nonhistone substrates (reviewed in Sterner and Berger 2000). Indeed, recent data indicate that such nonhistone substrates exist for NuA4 (Lin *et al.* 2009), including Yng2, which is also a substrate of Rpd3 (Lin *et al.* 2008). Further studies should provide additional insight into the range and roles of nonhistone substrates in Esa1 and Rpd3 functions, perhaps revealing a more direct link for their influence on rDNA and telomeric silencing. In this case however, we have shown that H4K12 is a key shared target for the contributions of Esa1 and Rpd3 to cell growth and viability.

A critical role for dynamic acetylation and deacetylation of H4K12 by Esa1 and Rpd3L: The cell contains numerous HATs and HDACs that together acetylate many lysine residues on histones. The intricacies of histone acetylation and deacetylation result from several features: each HAT and HDAC often targets multiple lysine residues, different HATs and HDACs have overlapping acetylation targets, and other post-translational modifications may influence activity or substrate recognition. For example, a simple mutation of H4K12 did not suppress *esa1* defects (Figure 6A), even though it is a key target of Esa1. This is because Esa1 has many other histone targets, including other lysines on H4, H2A, and H2A.Z, and lack of acetylation of these also contributes to *esa1*'s growth defect.

Defining roles for specific histone acetylation sites is further complicated by the genomewide data that acetylation of H4K5, H4K8, and H4K12 are redundant in transcription (Dion *et al.* 2005). One point in support of this idea is that the H4K12A single mutant displayed no obvious growth defects (Figure 6A). A previous study defined the H3 and H4 N termini as the functional targets of Rpd3 in regulation of transcription (Sabet *et al.* 2004). The connections between Esa1, Rpd3L, and H4K12 presented here strengthen this functional importance through further identification of a key specific lysine (H4K12) in the H4 N terminus, and the acetyltransferase responsible (Esa1). Since in the absence of Esa1 and Rpd3L, H4K12 acetylation became particularly important for cell viability (Figure 6), these specific links define a model whereby control of H4K12 acetylation is essential for transcriptional regulation of a subset of genes by Esa1 and Rpd3L for cell viability (Figure 7A).

Among the several genomewide ChIP data sets that define Esa1 and Rpd3 binding (Reid *et al.* 2000; Kurdistani *et al.* 2002; Robert *et al.* 2004), little overlap has been observed between regions strongly enriched for Esa1 and those enriched for Rpd3. This may be due to the fact that Esa1 and Rpd3 both exist in multiple complexes in the cell, creating noise in the data sets. Esa1-bound loci would include both NuA4 and piccolo, thereby conflating their occupancy sites. Likewise, genomewide ChIP that has been performed does not allow discrimination between sites of Rpd3L *vs.* Rpd3S occupancy. When analyzed at specific loci, Rpd3S functions at downstream regions (Carrozza *et al.* 2005b; Keogh *et al.* 2005), thus it is likely that the genomewide binding of Rpd3 found at downstream regions can be attributed to Rpd3S and binding at promoters can be attributed to Rpd3L. However, because the genomic binding maps were generated with nontiling arrays, resolving the differences in Rpd3L and Rpd3S binding with available data sets is not possible. The differences in function between Rpd3L and Rpd3S in relation to Esa1 provide a new tool for refining understanding of the two complexes.

Distinguishing complexes and their functional interactions: Esa1 and Rpd3 each act as the catalytic subunit of two multiprotein histone modifying complexes. The two Rpd3 complexes, Rpd3S and Rpd3L, are composed of distinct subunits that allow them to be genetically dissected. Several recent articles have examined different roles for Rpd3S and Rpd3L (Carrozza *et al.* 2005a,b; Keogh *et al.* 2005; Biswas *et al.* 2008; Knott *et al.* 2009). We have established that disruption of Rpd3L function is specifically responsible for the genetic suppression of *esa1* mutants for cell viability and silencing phenotypes (Figures 2 and 3).

Two different roles for histone modifying enzymes in the DNA damage response have been uncovered. One role is to participate in the transcriptional response through the activation of DNA repair genes. For example, Esa1 and Rpd3 are both required for transcriptional activation of the damage-inducible genes *HUG1* and *RNR3* (Sharma *et al.* 2007). The identification of these as shared targets for activation raises a useful distinction because *rpd3* cannot suppress *esa1*'s sensitivity to DNA damage. It seems likely therefore that Esa1 and Rpd3 target genes relevant to changes in H4K12 acetylation are those that Esa1 activates and

Rpd3 represses. Identification of these genes should prove of great interest.

The other function for histone modifying enzymes in DNA repair is more direct: chromatin modification targeted to the site of DNA damage. Along with several other HATs and HDACs, Esa1, some members of NuA4, and Rpd3 itself all bind at double-strand breaks, followed by changes in acetylation of nearby chromatin (Bird *et al.* 2002; Downs *et al.* 2004; Tamburini and Tyler 2005; Lin *et al.* 2008). Our observations showing that deletion of Rpd3L-specific subunits does not suppress repair defects of *esa1* mutants make it unlikely that Rpd3L functions together with Esa1 at sites of DNA damage. However, since Rpd3 is present at double-strand breaks and is required for nonhomologous end joining (Jazayeri *et al.* 2004; Tamburini and Tyler 2005), perhaps Rpd3S and Esa1 coordinate acetylation at sites of DNA damage (Figure 7C).

By constructing specific double deletion mutants, it was possible to refine understanding of Rpd3S and Rpd3L functions beyond earlier reports. In contrast, Esa1 exists in the NuA4 and piccolo complexes, yet because piccolo is a subcomplex of NuA4, it has not yet been possible to disrupt piccolo without also disrupting NuA4 function. Therefore, it remains to be determined whether *rpd3* suppression of *esa1* is mediated through NuA4 or piccolo.

Future studies should provide additional insight into distinctions between NuA4 and piccolo that may allow this question to be answered. One idea comes from studies examining the chromatin modifying complexes SLIK/SALSA and SAGA. These complexes share most subunits, including the histone acetyltransferase Gcn5. It was found that the shared subunit Spt7 exists in a C-terminally truncated form in the smaller SLIK/SALSA complex, allowing for construction of specific *SPT7* alleles that favor a specific complex (Sterner *et al.* 2002; Wu and Winston 2002). Analogous to this shift between SLIK/SALSA and SAGA, the discovery of specific alleles of piccolo components that favor activity of one complex over another may allow for future dissection of Esa1's interactions with Rpd3.

One possibility is that NuA4 and Rpd3S, which share the chomodomain protein Eaf3, work together, whereas piccolo and Rpd3L are also a functional pair. Some data supporting this idea can be extrapolated from genome-wide studies. For example, double mutants of *RPD3* and genes encoding NuA4-specific subunits show reduced fitness, whereas a double deletion mutant of *RPD3* and *EPL1*, which is in both piccolo and NuA4, shows synthetic rescue (Lin *et al.* 2008). However, because deletion of *EPL1* disrupts both piccolo and NuA4, it is difficult to make a clear distinction between the two.

The composition of NuA4, piccolo, Rpd3L, and Rpd3S is evolutionarily conserved (reviewed in Doyon and Côté 2004; Yang and Seto 2008). One particular class of proteins in both is the PHD finger-containing ING family of tumor suppressors. Yng2 is a yeast ING protein that is a subunit of both piccolo and NuA4 (Loewith *et al.* 2000), whereas Pho23 is another yeast ING protein that is a subunit of Rpd3L (Loewith *et al.* 2001; Carrozza *et al.* 2005a). The precise functions of Yng2 and Pho23 in their complexes are unknown, but analogous to the opposing activities between Esa1 and Rpd3, Yng2 and Pho23 have opposite effects on p53-dependent transcriptional activation, shown in an experiment where human p53 was expressed in yeast to drive transcription (Nourani *et al.* 2003). This opposing effect on activity of a human protein in transcription emphasizes the conserved nature underlying the partnership between the Esa1 and Rpd3 complexes reported here. In addition, the identification of H4K12 as a critical shared acetylation target uncovers the importance of the dynamic acetylation and deacetylation of a particular histone residue in the context of Esa1 and Rpd3L function.

Dynamic and reciprocal histone modifications are increasingly recognized as key regulatory switches. This principle was highlighted in a recent study investigating histone ubiquitination in metazoan development. Coordinate control of H2B ubiquitination in Drosophila by the ubiquitin ligase dBRE1 and the ubiquitin protease Scrawny was found to be essential for regulating gene silencing to promote cellular differentiation (Buszczak *et al.* 2009). Our studies identify links between Esa1 and Rpd3L specifically in the acetylation and deacetylation of H4K12. Further, they reveal a critical distinct characteristic of the Rpd3L complex in relation to Esa1, and identify roles for specific histone residues in promoting cell viability. Future functional dissection of Rpd3 and Esa1 multiprotein complexes will deepen understanding of how such chromatin modifiers control important and diverse cellular processes.

We thank E. M. Scott, B. Wood, S. Torigoe, M. Ruault, D. Stillman, M. Smith, and M. Grunstein for providing strains and reagents. We also thank R. M. Garza, S. J. Jacobson, M. R. Koch, M. Ruault, and E. M. Scott for critical reading of the manuscript, and members of the Pillus lab for advice throughout the course of this study. This work was supported by the National Institutes of Health and the University of California Cancer Research Coordinating Committee.

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Communicating editor: M. Hampsey

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Collaboration Between the Essential Esa1 Acetyltransferase and the Rpd3 Deacetylase Is Mediated by H4K12 Histone Acetylation in *Saccharomyces cerevisiae*

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DOI: 10.1534/genetics.109.103846

FILE S1

Supporting Materials and Methods

Yeast strains. Histone mutant strains with telomeric silencing reporter initially contained pLP2224 (*HHF2 HHT2 HIS3* CEN), and were transformed with *TRP1* plasmid carrying relevant H4 (*HHF2*) mutation. Loss of pLP2224 was screened on SC-His plates.

Qualitative mating assays. *MAT*a strains were patched onto YPD, grown up overnight at 30˚ and replica plated onto a minimal medium plate spread with a lawn of mating-type tester *MAT*α (LPY78). Plates were grown at 30˚ for 2-3 days prior to data collection.

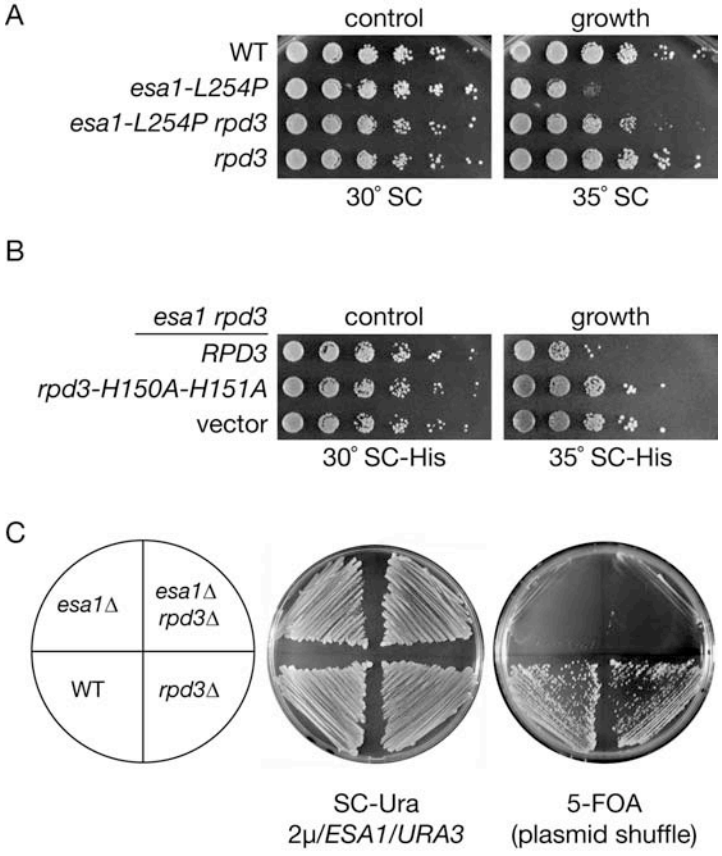

FIGURE S1.—(A) Suppression of *esa1*'s growth defect by *rpd3* was not allele-specific. Serial dilutions of wild-type (LPY5), *esa1-L254P* (LPY12160), *esa1-L254P rpd3* (LPY12164), and *rpd3* (LPY12154) were plated on SC at the permissive and restrictive temperatures. (B) *esa1 rpd3* carrying either wild-type *RPD3* (LPY14359), catalytically inactive *rpd3-H150A-H151A* (LPY14360), or vector (LPY14356) on a *HIS3* plasmid were plated at permissive and restrictive temperatures on SC-His to assay for growth. (C) Deletion of *RPD3* did not bypass the need for *ESA1*. The following strains, wild-type (LPY12200), *esa1*Δ (LPY12204), *esa1*Δ *rpd3* (LPY12206) and *rpd3* (LPY12202), all carried a wild-type *URA3/ESA1* plasmid (pLP796). These strains were subjected to a plasmid shuffle by counterselection on 5-FOA.

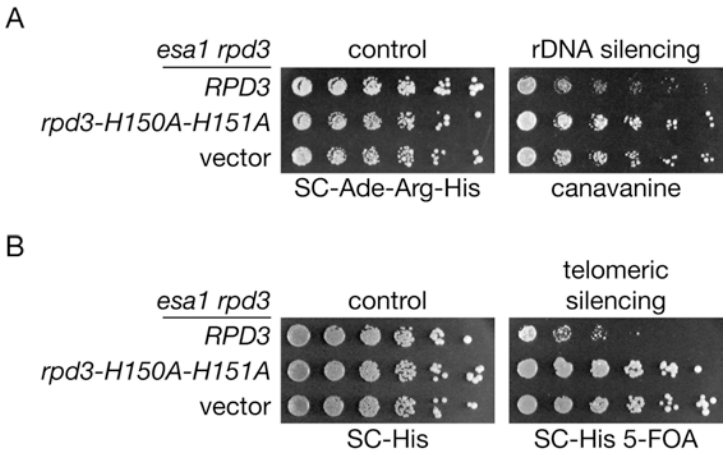

FIGURE S2.—*RPD3* mutant lacking catalytic activity suppresses silencing defects of *esa1*. (B) *esa1 rpd3* double mutant strains with rDNA::*ADE2-CAN1* reporter carrying either wild-type *RPD3* (LPY14660), *rpd3-H150A-H151A* (LPY14661), or vector (LPY14659), were assayed for rDNA silencing on SC-Ade-Arg-His plates with canavanine (32 μg/mL). (C) *esa1 rpd3* with TELVR::*URA3* carrying wild-type *RPD3* (LPY14365), *rpd3-H150A-H151A* (LPY14366), and vector (LPY14364), were assayed for telomeric silencing on SC-His 5-FOA plates.

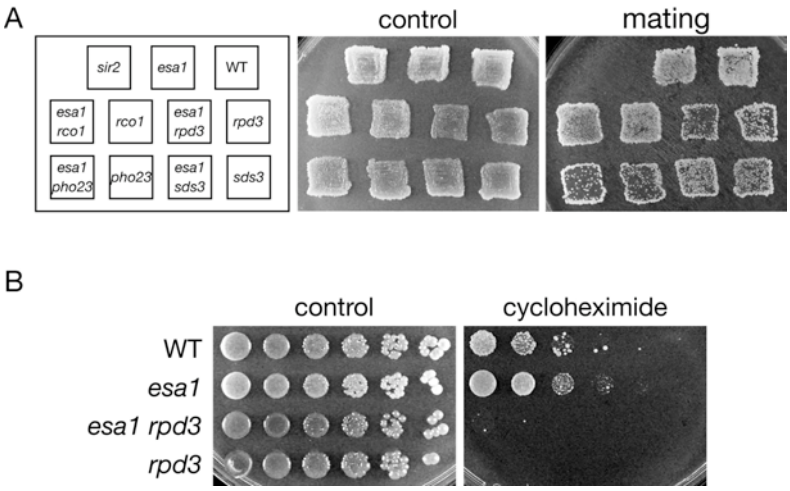

FIGURE S3.—Mutant phenotypes of *rpd3* are not suppressed by *esa1* mutation. (A) Mutation of *ESA1* had no effect on *rpd3*'s mating defect. Single and double mutants from Figure 1 and Figure 2 were patched onto YPD and mated to tester α strain (LPY78) by replica plating. A non-mating *sir2* strain (LPY11) was used as a control. (B) Mutation of *ESA1* had no effect on *rpd3*'s cycloheximide sensitivity. Wild-type (LPY5), *esa1* (LPY4774), *esa1 rpd3* (LPY12156), and *rpd3* (LPY12154) strains were tested for growth on YPD plates containing 250 ng/mL cycloheximide.

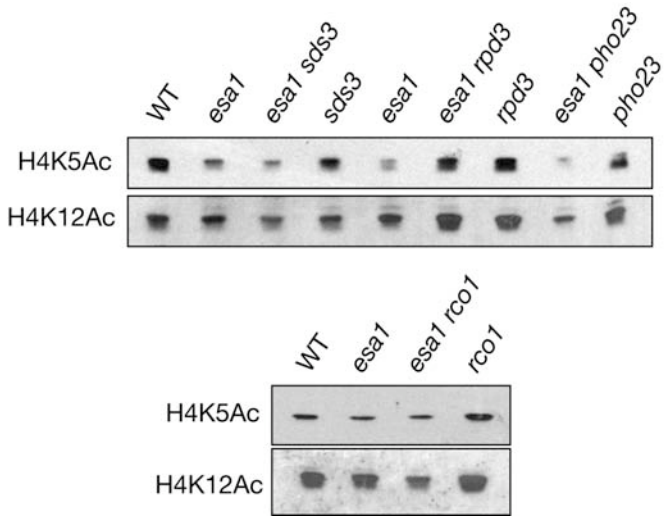

FIGURE S4.—Deletion of non-catalytic subunits of Rpd3 complexes does not change global acetylation of H4K5 or H4K12. Whole cell extracts were prepared from strains in Figure 2 as described in Figure 5 and immunoblotted for global acetylation changes at H4K5 and H4K12.

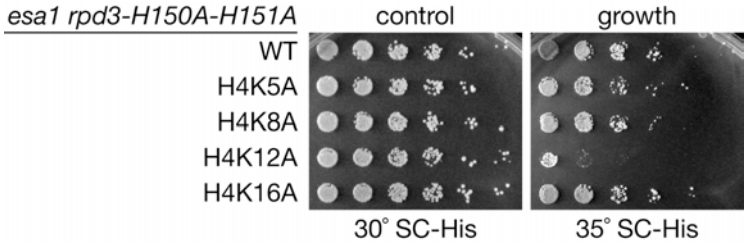

FIGURE S5. —Growth rescue of *esa1* mutant by catalytcally inactive *rpd3-H150A-H151A* is dependent on H4K12. *esa1 rpd3* strains carrying different H4 lysine mutant plasmids were transformed with a *HIS3* plasmid containing *rpd3-H150A-H151A.* These were plated on SC-His at permissive and restrictive temperatures to assay for growth. All strains are *esa1 rpd3-H150A-H151A* with either wild-type H4 (LPY14675), H4K5A (LPY14676), H4K8A (LPY14677), H4K12A (LPY14678), or H4K16A (LPY14679).

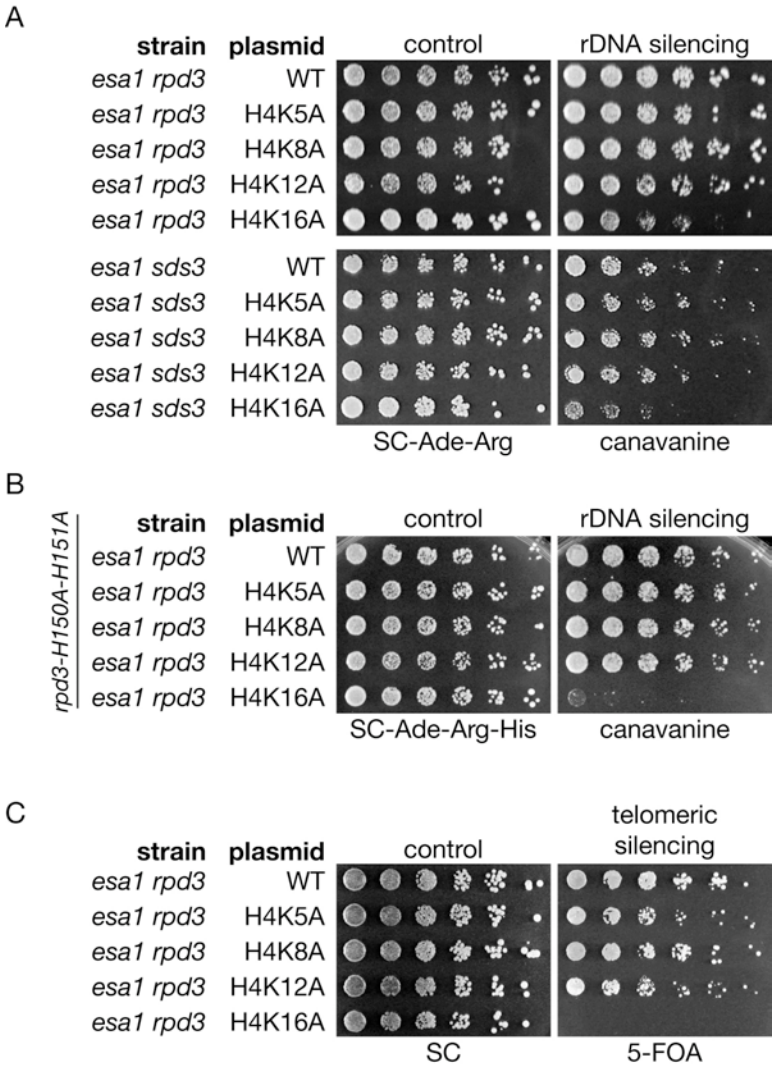

FIGURE S6.—rDNA and telomeric silencing suppression is not dependent on a single H4 lysine. (A) Top panel: same strains from Figure 6A were plated on SC-Ade-Arg with 32 μg/ml canavanine to test for rDNA silencing defects with the rDNA::*ADE2-CAN1* reporter. Bottom panel: *esa1 sds3* (LPY14296), *esa1 sds3* H4K5A (LPY14297), *esa1 sds3* H4K8A (LPY14298), *esa1 sds3* H4K12A (LPY14299), *esa1 sds3* H4K16A (LPY14300) all have the rDNA::*ADE2-CAN1* silencing reporter, and were assayed as above. (B) Strains from part (A) top panel, were transformed with catalytically-inactive *rpd3-H150-H151A* (transformed strains are LPY14685, LPY14686, LPY14687, LPY14688, and LPY14689, in order) and assayed for silencing on SC-Ade-Arg-His plates with 32 μg/ml canavanine. (C) *esa1 rpd3* (LPY14301), *esa1 rpd3* H4K5A (LPY14302), *esa1 rpd3* H4K8A (LPY14303), *esa1 rpd3* H4K12A (LPY14304), *esa1 rpd3* H4K16A (LPY14305) were plated on 5-FOA plates to test for telomeric silencing using the TELVR::*URA3* silencing marker.

TABLE S1

Yeast strains used in this study[a]

Strain	Genotype	Reference
LPY5 (W303-1a)	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
LPY11	W303 *MAT*a *sir2::HIS3*	
LPY78	*MAT*α *his4*	P. Schatz
LPY4774	W303 *MAT*a *esa1-414*	
LPY4909	W303 *MAT*α rDNA*::ADE2-CAN1*	Clarke et al. 2006
LPY4911	W303 *MAT*α *esa1-414* rDNA*::ADE2-CAN1*	Clarke et al. 2006
LPY4917	W303 *MAT*α TELVR*::URA3*	Clarke et al. 2006
LPY4919	W303 *MAT*α *esa1-414* TELVR*::URA3*	Clarke et al. 2006
LPY11160	W303 *MAT*α *esa1-414 sir2::HIS3*	
LPY11816	W303 *MAT*α *hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* rDNA*::ADE2-CAN1* + pJH33	
LPY11817	W303 *MAT*α *esa1-414 hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* rDNA*::ADE2-CAN1* + pJH33	
LPY12071	LPY11817 + pLP2146 (no pJH33)	
LPY12093	W303 *MAT*α *rpd3::kanMX* TELVR*::URA3*	
LPY12145	W303 *MAT*α *rpd3::kanMX* rDNA*::ADE2-CAN1*	
LPY12147	W303 *MAT*α *esa1-414 rpd3::kanMX* rDNA*::ADE2-CAN1*	
LPY12154	W303 *MAT*a *rpd3::kanMX*	
LPY12156	W303 *MAT*a *esa1-414 rpd3::kanMX*	
LPY12160	W303 *MAT*a *esa1-L254P*	
LPY12164	W303 *MAT*a *esa1-L254P rpd3::kanMX*	
LPY12200	W303 *MAT*a + pLP796	
LPY12202	W303 *MAT*a *rpd3::kanMX* + pLP796	
LPY12204	W303 *MAT*a *esa1*Δ*::HIS3* + pLP796	
LPY12206	W303 *MAT*a *esa1*Δ*::HIS3 rpd3::kanMX* + pLP796	
LPY12211	W303 *MAT*α *esa1-414 rpd3::kanMX* TELVR*::URA3*	
LPY12228	W303 *MAT*α *rpd3::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* rDNA*::ADE2-CAN1* + pJH33	
LPY12230	W303 *MAT*α *esa1-414 rpd3::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* rDNA*::ADE2-CAN1* + pJH33	
LPY12236	W303 *MAT*a *esa1-414 rpd3::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* + pJH33	
LPY12383	LPY11816 + pLP1775 (no pJH33)	
LPY12384	LPY11817 + pLP1775 (no pJH33)	
LPY12394	LPY11816 + pLP2146 (no pJH33)	
LPY12645	W303 *MAT*a *rco1*Δ*::kanMX*	
LPY12652	W303 *MAT*a *esa1-414 rco1*Δ*::kanMX*	

LPY12695	LPY12228 + pLP1775 (no pJH33)
LPY12702	LPY12228 + pLP2146 (no pJH33)
LPY12707	LPY12230 + pLP1775 (no pJH33)
LPY12708	LPY12230 + pLP2181 (no pJH33)
LPY12711	LPY12230 + pLP2145 (no pJH33)
LPY12714	LPY12230 + pLP2146 (no pJH33)
LPY12717	LPY12230 + pLP1990 (no pJH33)
LPY12729	W303 *MAT*a *esa1-414 pho23*Δ*::kanMX*
LPY12732	W303 *MAT*a *pho23*Δ*::kanMX*
LPY12956	W303 *MAT*a *esa1-414 sds3*Δ*::kanMX*
LPY12958	W303 *MAT*a *sds3*Δ*::kanMX*
LPY13124	W303 *MAT*α *esa1-414 sds3*Δ*::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* rDNA*::ADE2-CAN1* + pJH33
LPY13139	W303 *MAT*a *esa1-414 pho23*Δ*::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* + pJH33
LPY13183	W303 *MAT*a *esa1-414 sds3*Δ*::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* + pJH33
LPY13472	W303 *MAT*a *hda1::kanMX*
LPY13478	W303 *MAT*a *esa1-414 hda1::kanMX*
LPY13501	W303 *MAT*α *rco1*Δ*::KanMX* rDNA*::ADE2-CAN1*
LPY13505	W303 *MAT*α *esa1-414 rco1*Δ*::kanMX* rDNA*::ADE2-CAN1*
LPY13513	W303 *MAT*α *sds3*Δ*::kanMX* rDNA*::ADE2-CAN1*
LPY13517	W303 *MAT*α *esa1-414 sds3*Δ*::kanMX* rDNA*::ADE2-CAN1*
LPY13524	W303 *MAT*α *rco1*Δ*::kanMX* TELVR*::URA3*
LPY13528	W303 *MAT*α *esa1-414 rco1*Δ*::kanMX* TELVR*::URA3*
LPY13536	W303 *MAT*α *sds3*Δ*::kanMX* TELVR*::URA3*
LPY13540	W303 *MAT*α *esa1-414 sds3*Δ*::kanMX* TELVR*::URA3*
LPY13583	W303 *MAT*a *hos2::kanMX*
LPY13585	W303 *MAT*a *esa1-414 hos2::kanMX*
LPY13706	W303 *MAT*a *hos1::kanMX*
LPY13712	W303 *MAT*a *esa1-414 hos1::kanMX*
LPY13765	W303 *MAT*α *pho23*Δ*::kanMX* TELVR*::URA3*
LPY13769	W303 *MAT*α *esa1-414 pho23*Δ*::kanMX* TELVR*::URA3*
LPY13854	W303 *MAT*α *pho23*Δ*::kanMX* rDNA*::ADE2-CAN1*
LPY13859	W303 *MAT*α *esa1-414 pho23*Δ*::kanMX* rDNA*::ADE2-CAN1*
LPY14165	LPY13139 + pLP1775 (no pJH33)
LPY14166	LPY13139 + pLP2181 (no pJH33)
LPY14167	LPY13139 + pLP2145 (no pJH33)
LPY14168	LPY13139 + pLP2146 (no pJH33)
LPY14169	LPY13139 + pLP1990 (no pJH33)
LPY14175	LPY13183 + pLP1775 (no pJH33)

LPY14176	LPY13183 + pLP2181 (no pJH33)
LPY14177	LPY13183 + pLP2145 (no pJH33)
LPY14178	LPY13183 + pLP2146 (no pJH33)
LPY14179	LPY13183 + pLP1990 (no pJH33)
LPY14247	W303 *MAT*a *esa1-414 rpd3::kanMX hht1-hhf1*Δ*::kanMX hht2-hhf2*Δ*::kanMX hta2-htb2*Δ*::HPH* TELVR*::URA3* + pLP2224
LPY14296	LPY13124 + pLP1775 (no pJH33)
LPY14297	LPY13124 + pLP2181 (no pJH33)
LPY14298	LPY13124 + pLP2145 (no pJH33)
LPY14299	LPY13124 + pLP2146 (no pJH33)
LPY14300	LPY13124 + pLP1990 (no pJH33)
LPY14301	LPY14247 + pLP1775 (no pLP2224)
LPY14302	LPY14247 + pLP2181 (no pLP2224)
LPY14303	LPY14247 + pLP2145 (no pLP2224)
LPY14304	LPY14247 + pLP2146 (no pLP2224)
LPY14305	LPY14247 + pLP1990 (no pLP2224)
LPY14356	LPY12156 + pLP60
LPY14359	LPY12156 + pLP1945
LPY14360	LPY12156 + pLP1946
LPY14364	LPY12211 + pLP60
LPY14365	LPY12211 + pLP1945
LPY14366	LPY12211 + pLP1946
LPY14659	LPY12147 + pLP60
LPY14660	LPY12147 + pLP1945
LPY14661	LPY12147 + pLP1946
LPY14675	LPY12236 + pLP1775 + pLP1946 (no pJH33)
LPY14676	LPY12236 + pLP2181 + pLP1946 (no pJH33)
LPY14677	LPY12236 + pLP2145 + pLP1946 (no pJH33)
LPY14678	LPY12236 + pLP2146 + pLP1946 (no pJH33)
LPY14679	LPY12236 + pLP1990 + pLP1946 (no pJH33)
LPY14685	LPY12707 + pLP1946
LPY14686	LPY12708 + pLP1946
LPY14687	LPY12711 + pLP1946
LPY14688	LPY12714 + pLP1946
LPY14689	LPY12717 + pLP1946

[a]Unless otherwise noted, strains were constructed during the course of this study or are part of the standard lab collection.

TABLE S2

Plasmids used in this study[b]

Plasmid (alias)	Description	Source/Reference
pJH33	*HTA1 HTB1 HHF2 HHT2 URA3* CEN	Ahn et al. 2005
pLP1775	*HHF2 HHT2 TRP1* CEN	S. L. Berger
pLP2181	*hhf2-K5A HHT2 TRP1* CEN	
pLP2145	*hhf2-K8A HHT2 TRP1* CEN	
pLP2146	*hhf2-K12A HHT2 TRP1* CEN	
pLP1990	*hhf2-K16A HHT2 TRP1* CEN	
pLP2224	*HHF2 HHT2 HIS3* CEN	
pLP796	*ESA1 URA3* 2μ	Clarke et al. 2006
pRS313 (pLP60)	vector *HIS3* CEN	Sikorski and Hieter 1989
pLP1945	*RPD3*-13myc *HIS3* CEN	Ruault and Pillus 2006
pLP1946	*rpd3-H150A-H151A*-13myc *HIS3* CEN	Ruault and Pillus 2006

[b]Unless otherwise noted, plasmids were constructed during the course of this study or are part of the standard lab collection.

TABLE S3

Oligonucleotide sequences used in this study[c]

Oligo #	Name	Sequence (5'-3')	Source/Reference
921	H4K5A sense	TCCGGTAGAGGT**GC**AGGTGGTAAAGG	
922	H4K5A antisense	CCTTTACCACCT**GC**ACCTCTACCGGA	
898	H4K8A sense	GTAAAGGTGGT**GC**AGGTCTAGGAAAAGG	
899	H4K8A antisense	CCTTTTCCTAGACCT**GC**ACCACCTTTAC	
900	H4K12A sense	AAGGTCTAGGA**GC**AGGTGGTGCCAAGC	
901	H4K12A antisense	GCTTGGCACCACCT**GC**TCCTAGACCTT	
788	H4K16A sense	GGAAAAGGTGGTGCC**GC**GCGTCACAGAAAGATT	
789	H4K16A antisense	AATCTTTCTGTGACGC**GC**GGCACCACCTTTTCC	
950	*PHO23* KO forward	CTTCGCCCAGCACATTGTCC	
951	*PHO23* KO reverse	CGGCGATTAGACTGAGCTGC	
974	*SDS3* KO forward	CACTCAAGCGATGATCGTTTCG	
975	*SDS3* KO reverse	CTACAGTGGCATTAGTTGCAGC	
956	*RCO1* KO forward	GCCAATCTGGCTTCCCTAATAGC	
957	*RCO1* KO reverse	GGCAACATTCAGCATATCCAGG	

[c]Nucleotides in **bold** in the above sequences are mutagenic, compared to the wild-type sequence.

TABLE S4

Summary of Results: effects of second mutation on *esa1* mutant

esa1 phenotype	*rpd3*Δ§	*rpd3-H150A-H151A*§	*sds3*Δ‡	*pho23*Δ‡	*rco1*Δ†
growth suppression (dependence on K12)	+ (yes)	+ (yes)	+ (yes)	+ (yes)	–
rDNA silencing (dependence on K12)	+ (no)	+ (no)	+ (no)	+	–
TEL silencing (dependence on K12)	+ (no)	+	+	+	–
CPT suppression	–		–	–	–/+
restore global H4Ac	+		–/+	–/+	–/+

+ indicates suppression of *esa1* phenotype
– indicates exacerbation of *esa1* phenotype
–/+ indicates no change
§ catalytic (Rpd3) subunit
‡ Rpd3L subunit
† Rpd3S subunit